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(72) Inventor: **Kyle, David John**

**Catonsville, MD 21228 (US)**

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(74) Representative: **Mercer, Christopher Paul et al**

**Carpmaels & Ransford,  
43-45 Bloomsbury Square  
London WC1A 2RA (GB)**

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(71) Applicant: **Martek Biosciences Corporation**  
**Columbia, MD 21045 (US)**

(54) **Use of docosahexaenoic acid for the manufacture of a medicament for the treatment of senile dementia and Alzheimer's disease**

(57) The present invention provides the use of a composition comprising docosahexaenoic acid (DHA), in the manufacture of a medicament for the treatment, alleviation or prevention of the symptoms of an ailment

selected from senile dementia and Alzheimer's disease, characterised in that the composition is substantially free of eicosapentaenoic acid (EPA).

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## Description

### FIELD OF THE INVENTION

[0001] This invention relates to a method of treating neurological disorders, including certain neurodegenerative diseases and psychiatric disorders, by administering a composition comprising a therapeutically effective amount of a single cell microbial oil comprising docosahexaenoic acid (DHA), a single cell oil comprising arachidonic acid (ARA) or a combination of DHA- and ARA-containing oils, to a person in need of such treatment. The oils can be administered as a pharmaceutical composition, as a dietary supplement, or in the form of a food product by replacing a portion of the vegetable oil or fat thereon.

### BACKGROUND OF THE INVENTION

[0002] The human brain and other neural tissues are highly enriched in long chain polyunsaturated fatty acids which are thought to play an important role in modulating the structure, fluidity and function of the cell membranes of these tissues. Arachidonic acid (hereafter referred to as ARA) is a long chain polyunsaturated fatty acid of the  $\omega$ -6 class (5, 8, 11, 14-eicosatetraenoic acid, 20:4 $\omega$ -6), and is the most abundant C<sub>20</sub> polyunsaturated fatty acid in the human body. In addition to its primary role as a structural lipid, ARA also is the direct precursor for a number of circulating eicosenoids such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) prostacyclin I<sub>2</sub> (PGI<sub>2</sub>) thromboxane A<sub>2</sub> (TXA<sub>2</sub>), and leukotrienes B<sub>4</sub> (LTB<sub>4</sub>) and C<sub>4</sub> (LTC<sub>4</sub>). These eicosenoids exhibit regulatory effects on lipoprotein metabolism, blood rheology, vascular tone, leukocyte function and platelet activation. In humans ARA is not synthesized de novo, but it can be synthesized by the elongation and desaturation of linoleic acid, an essential fatty acid which must be obtained from the diet.

[0003] Docosahexaenoic acid (4, 7, 10, 13, 16, 19-docosahexaenoic acid 22:6 $\omega$ -3) (hereinafter referred to as DHA) is the most abundant of the fatty acids of the structural components of grey matter of the human brain and other neural tissues. DHA cannot be synthesized de novo in humans, but there is some evidence that this  $\omega$ -3 fatty acid can be synthesized by some cell types, such as astrocytes, if the appropriate long chain polyunsaturated fatty acids are provided in the diet. S. Moore et al., *Journ. of Neurochemistry* 56 (1991) pgs. 518 to 524. Most of the DHA found in the brain and retina cell membranes is believed to be obtained from dietary sources.

[0004] The importance of providing polyunsaturated fatty acids during a period of rapid brain development to prevent irreparable damage to brain cells is well known in the art. Human infants appear to have a particularly poor ability to synthesize DHA, but any deficiencies can be compensated for by feeding infants human breast milk, which is a rich source of essential fatty acids, particularly DHA and ARA. Sanders et al., *Am. J. Clin. Nutr.*,

31 (1978) pgs. 805-813. Recent studies comparing the performance on standard intelligence tests of children who were fed breast milk as babies to children who were fed commercial infant formulas as babies have suggested a dose response relationship between the proportion of mother's milk in the diet and subsequent IQ. A. Lucas, R. Morley, T.J. Cole, G. Lister, and C. Leeson-Payne *Lancet* 339 (1992) pgs. 261-264. These studies suggest that dietary intervention therapy can effect the levels of DHA available for structural development of the nervous system.

[0005] It has been observed that DHA levels in two major classes of phospholipid, phosphatidylethanolamine and phosphatidylcholine, are significantly reduced in the brain tissues of patients with Alzheimer's disease. Control samples taken from patients of advanced age, having no clinical manifestations of dementia or other mental disorders showed no significant changes in the fatty acid composition of these two classes of phospholipids. These results suggest that the alterations in DHA concentrations in the brain tissue of Alzheimer's patients are not the result of normal aging, but are specific for the pathological mechanisms involved in this neurodegenerative disease. M. Soderberg, C. Edlund, K. Kristensson and G. Daliner, *Lipids* 26 (1991) pgs. 421-425.

[0006] Peroxisomal disorders are a group of degenerative neurological disorders characterized by increased levels of very long chain fatty acids, resulting from an impaired capacity of the effected individuals for degrading these fatty acids. These disorders are related in that they all appear to result from some defect localized in the subcellular organelles known as peroxisomes. N. Gordon "Peroxisomal Disorders", *Brain Development* 9 (1987) pgs. 571-575. These peroxisomal disorders have been classified into three groups based on the extent of the loss of peroxisomal functions found in a particular disease. A. C. Theil, *European Journal of Pediatrics*, 151 (1992) pgs. 117-120.

[0007] The group 1 peroxisomal disorders are characterized by a virtually complete loss of peroxisomes and peroxisomal functions. These disorders include Zellweger's syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease and hyperpepelic acidemia. The group 2 disorders are characterized by the loss of multiple peroxisomal functions and include Rhizomelic chondrodysplasia punctata and Zellweger-like syndrome. The group 3 disorders are characterized by the loss of only a single peroxisomal function and include adrenoleukodystrophy, adrenomyeloneuropathy, acyl-CoA oxidase deficiency, bifunctional protein deficiency, thiolase deficiency, hyperoxaluria type I, acatalasaemia and adult Refsum disease. Clinical presentation of patients with peroxisomal disorders shows a wide divergence in phenotypic expression which varies significantly depending upon the patient's age. However, in all patients neurological functions are progressively impaired, which often leads to deterioration of the auto-

nostic functions and death at an early age.

[0008] Recent studies of the polyunsaturated fatty acid composition of tissues in patients with peroxisomal disorders have shown that, even though the total amount of fatty acids in these tissues was normal, there are significant changes in the fatty acid composition of the patient's tissues. These patients have a significant decrease in the total amount of DHA and ARA in their serum lipid compositions. Serum plasmalogen levels are also depressed.

[0009] Usher's syndrome is an autosomal recessive genetic disorder which is associated with the degeneration of visual cells, causing retinitis pigmentosa. The visual cells contain extremely large quantities of DHA esterified in the phospholipids of the photoreceptor membranes which make up the outer segments of the visual cells. Bazan and coworkers recently have found that the plasma phospholipids of Usher's patients contain significantly less DHA and ARA than the plasma phospholipids of unaffected individuals. N. G. Bazan, B. L. Scott, T. S. Reddy and M. Z. Pelias, *Biochem. Biophys. Res. Comm.* 141 (1986) pgs. 600-604.

[0010] In addition researchers have found that patients suffering from other clinical conditions, such as senile dementia, diabetes-induced neuropathy, multiple sclerosis, schizophrenia and neuropathies associated with high doses of heavy metals such as lead, aluminum, and mercury also frequently have levels of DHA and/or ARA in their serum lipids which are significantly depressed in comparison to the levels found in healthy persons. For example, recent studies have established a correlation between alterations in the levels of esterification of ARA into the phospholipids of platelets and the presence of schizoaffective disorders in patients. L. Demisch et al., *Prostaglandins Leukot. Essent. Fatty Acids* 46 (1992) pgs. 47-52. Evidence of abnormal essential fatty acid biochemistry in the plasma phospholipids of patients with schizophrenia also has been reported. D. F. Horrobin, *Prostaglandins Leukot. Essent. Fatty Acids* 46 (1992) pgs. 71-77.

[0011] Although researchers have made some progress in understanding neurodegenerative disorders such as Alzheimer's disease and various peroxisomal disorders, effective means of treating these disorders have remained elusive. Likewise, there has been a lack of progress in the development of effective therapeutic drugs to treat schizophrenia and other neurological disorders disclosed above.

[0012] Accordingly, it is an object of the present invention to provide a method for treating neurological disorders, in which the serum, tissue or membrane levels of the essential fatty acids DHA and ARA are affected.

#### SUMMARY OF THE INVENTION

[0013] This invention relates to a method of treating a patient suffering from a neurological disease, which comprises administering to the patient an effective

amount of the fatty acids DHA or ARA, or a mixture of DHA and ARA. These fatty acids are administered in the form of oils in which DHA and ARA are provided as natural complex lipids, preferably in the form of triglycerides. The neurological diseases to be treated include the group of diseases classified as peroxisomal diseases, Alzheimer's disease, and Usher's syndrome, senile dementia, diabetes-induced neuropathy, multiple sclerosis, schizophrenia and neuropathies associated with high doses of heavy metals such as lead, aluminum, and mercury, as well as other neurodegenerative diseases in which the serum, tissue or membrane concentrations of DHA or ARA are significantly affected in comparison to the DHA and ARA concentrations found in normal individuals. The invention also relates to pharmaceutical compositions containing DHA or ARA or to a composition containing both DHA and ARA which provide therapeutically effective amounts of these  $\omega$ -3 and  $\omega$ -6 fatty acids. Administration of these compositions provides prophylactic, as well as therapeutic, treatment of patients diagnosed with neurodegenerative disorders or with other DHA and ARA deficiency-related disorders such as schizophrenia. These methods of treatment and compositions also provide a prophylactic treatment for individuals who are at risk for developing one of these neurological disorders.

#### Detailed Description of the Invention

[0014] In accordance with the present invention, a method for treating a neurological disorder comprises administering to a person suffering from such a disorder a microbial oil comprising DHA, a microbial oil comprising ARA or a combination thereof. These neurological disorders include neurodegenerative disorders and certain psychiatric disorders such as schizophrenia, in which the serum, tissue or membrane levels of the essential fatty acids DHA and ARA are affected. The DHA and ARA are in the form of natural complex lipids. Preferably the DHA and ARA are in the form of triglycerides, although they also may be in the form of phospholipids. They are obtained as single cell microbial oils by the cultivation of DHA-producing microorganisms or ARA-producing organisms under oil-producing conditions.

[0015] According to preferred embodiments of the present invention, microorganisms capable of producing a single cell microbial oil containing DHA or ARA are cultivated in a fermentor in a nutrient solution capable of supporting the growth of such organisms. Preferably the microbial oil produced is enriched in the fatty acids of interest, meaning that it will contain at least about 20% DHA or 10% ARA by weight.

[0016] Any microorganism capable of producing a microbial oil containing DHA or ARA can be used in the present invention. These microorganisms can be identified by determining whether DHA or ARA oils are present in the fatty acid profiles of the harvested biomass from a culture of the microorganism. These pro-

files are typically obtained by gas chromatography of methyl ester derivatives of the fatty acids present in a sample.

[0017] As used herein, the term microorganism, or any specific type of microorganism, includes wild type strains, mutant strains or recombinant strains. Wild-type and recombinant microorganisms designed to produce microbial cell oil containing DHA or ARA can be used to produce the DHA-containing and ARA-containing microbial oils. Such recombinant strains would include those designed to produce greater quantities of DHA or ARA in the single cell oil, greater quantities of total oil, or both, as compared to the quantities produced by the same wild type microorganism, when provided with the same substrates. Microorganisms selected or designed to efficiently use more cost-effective substrates, while producing the same amount of single cell oil containing DHA or ARA as the wild-type microorganism, are particularly useful for preferred embodiments of the present invention.

[0018] For the production of DHA-containing microbial oils, species of photosynthetic algae such as *Chattonella*, *Skeletonema*, *Thalassiosira*, *Isochrysis*, *Hymenomonas*, or *Cryptomonas* can be used. Preferred microorganisms are heterotrophic species of algae which include, but are not limited to, the *Dinophyceae*, for example, *Cryptocodinium*; or to fungi such as *Chytridiomycetes*, for example, *Thraustochytrium*, or *Schizochytrium* or to the *Oomycetes*, for example, *Mortierella*, *Saprolegnia* or *Mucor*.

[0019] Preferred microorganisms for producing DHA are dinoflagellates, including *Cryptocodinium*. Especially preferred is *Cryptocodinium cohnii*, an obligate heterotroph, which is described in U.S. Application Serial No. 479,135, filed February 13, 1990. *C. cohnii* is preferred because it produces fatty acids in which DHA is the only polyunsaturated fatty acid present in quantities greater than about 1% of the total amount of polyunsaturated fatty acids, a quantity which is significant for carrying out the methods of the present invention. Samples of one strain of *C. cohnii*, which produces abundant levels of DHA, have been deposited with the American Type Culture Collection at Rockville, Maryland, and assigned ATCC accession number 40750.

[0020] Microorganisms useful for producing ARA include species of algae such as *Porphyridium*, *Ochromonas* and *Euglena*, and fungi such as *Pythium* and *Mortierella*. Many of those species which make ARA also produce significant quantities of eicosapentaenoic acid (EPA) in addition. Unexpectedly, it has been found that *P. insidiosum* and *M. alpina* produce ARA but are at least substantially free of EPA. "Substantially free" is defined to mean the ratio of ARA to EPA is at least 5:1. Preferably, the ratio is at least 10:1. Most desirably, no more than 1% of the fatty acid content of the oil is EPA. As with fish oils, high EPA levels in dietary supplements result in a depression of the ability to form ARA from dietary linoleic acid. Furthermore, the administration of

EPA-containing fish oils to patients, especially elderly, hypertensive or pregnant patients who may have increased prothrombin times, is undesirable because of the blood thinning effects of EPA. Accordingly, while those fungal species producing both ARA and EPA can be utilized in the process of this invention, it is preferable to use species which do not produce significant quantities of EPA. Preferred species include *Pythium insidiosum* and *Mortierella alpina*. Both species are available commercially and strains are on deposit with the American Type Culture Collective in Rockville, Maryland, such as those having ATCC accession numbers 28251 and 42430, respectively.

[0021] Likewise, although microbial species producing both DHA and EPA can be utilized as a source of the DHA oil used in this invention, it is preferable to use species which are at least substantially free of EPA. Preferably, the ratio is at least 10:1. Most desirably, no more than 1% of the fatty acid content of the oil is EPA.

#### Production of DHA-Containing Oil

[0022] The DHA-producing microorganisms can be cultivated in a simple medium containing a carbon source such as glucose and a nitrogen source such as yeast extract or peptone. Use of these components in a solution such as seawater provides economically significant growth rates and cell densities. During the course of a 3 to 5 day fermentation, for example, *C. cohnii* cell densities of at least 10 grams of biomass per liter of solution, and preferably from 20 to about 40 grams per liter, can be attained.

[0023] Although cultivation can occur in any suitable fermentor, preferably the organism is grown either in a stirred tank fermentor or in an air lift fermentor. When a stirred tank fermentor is selected, agitation is provided using either Rushton-type high efficiency turbines or pitched-blade or marine impellers. Agitation and sparging renew the supply of oxygen to the microorganisms. The rate of agitation normally is increased as the biomass increases, due to the increased demand for oxygen. It is desirable to keep the tip speed at not greater than about 500 cm per sec, preferably not greater than about 300 cm per sec. Selection of strains of microorganisms which are capable of withstanding greater tip speeds without undergoing shear damage is within the purview of those of skill in the art.

[0024] The organisms used for the production of DHA-containing oil can be grown in any suitable nutrient solution. As noted above, seawater is an acceptable medium for the nutrient solution for many organisms. The seawater can be either natural, filtered or an artificial mix, each of which can be diluted with water to reduced salinities, such as 1/2 to 1/4 normal strength, or concentrated to 2 times normal strength. A preferred medium is Instant Ocean® brand artificial seawater, or alternatively a mixture of 4.5 to 20 g per liter NaCl, 1.23 g per liter MgSO<sub>4</sub>·7H<sub>2</sub>O and 90 mg per liter CaCl<sub>2</sub> in wa-

ter. Micronutrients can be added and may be required when using defined media. However, such micronutrients are known to those of skill in the art and generally are present in seawater or tap water. If the organism selected is heterotrophic, such as *Cryptocodinium* and *Thraustochytrium*, then a reduced carbon source is added. *Cryptocodinium* and *Thraustochytrium* require a reduced carbon source for growth.

[0025] Preferably, after addition of the seawater medium to the fermentor, the fermentor containing the medium is sterilized and cooled prior to adding the nutrients and a seed culture of the microorganism to be cultivated. Although it is acceptable to sterilize the nutrients together with the seawater, sterilization in this manner can result in a loss of available glucose. The nutrients and microorganism can be added simultaneously or sequentially.

[0026] An effective seed concentration can be determined by those of skill in the art. When a stirred tank fermentor is used, the addition of a population of from about 0.05 to 1.0 grams of dry weight equivalent per liter at the beginning of the fermentation is preferred. For example, at least about 1 to  $5 \times 10^6$  cells of *C. cohnii* per ml would be suitable. Thus, for a 30 liter fermentor, 1 to 3 liters of seeding media, containing viable cells at a density of 10 to 20 grams dry weight per liter would be added.

[0027] Oxygen levels preferably are maintained at a dissolved oxygen of at least about 10% of air saturation level. Biosynthesis of DHA requires oxygen and, accordingly, higher yields of DHA require dissolved oxygen levels at from about 10% to 50% of air saturation levels. For example, agitation tip speeds of 150 to 200 cm per sec in combination with an aeration rate of 1 volume of air per volume of fermentor per minute (VVM) provides dissolved oxygen levels of from about 20% to about 30% at biomass densities of about 25 grams dry weight per liter of culture for *C. cohnii*. Higher cell densities may require higher dissolved oxygen levels, which can be attained by increased aeration rates by  $O_2$  sparging or by increasing the air pressure in the fermentor.

[0028] Acceptable carbon sources are known to those of skill in the art. For example, carbon can be provided to in the form of mono or di-saccharides such as sucrose, lactose, fructose or glucose. Autotrophs utilize carbon dioxide as a carbon source. Many organisms also will grow on other reduced, more complex, carbon sources, such as molasses, high fructose corn syrup and hydrolyzed starch. Typically, a fermentation is initiated with about 20 to 50 grams per liter glucose. More glucose is added during the fermentation as required. Alternatively, from about 50 to 150 grams glucose per liter initially can be added, thereby minimizing the frequency of future additions. The amount of carbon source provided to other organisms can readily be determined by those of skill in the art.

[0029] In addition to a reduced carbon source, a nitrogen source, such as yeast extract or peptone, is pro-

vided to the medium. For example, Difco or Marcor brand yeast extract and Shefftone brand peptone can be used. Yeast extract and peptone are organic nitrogen sources which also contain micronutrients. Other nitrogen sources easily can be determined by those of skill in the art. However, such compounds are generally more expensive than yeast extract. Any DHA- or ARA-producing algae strain variant capable of using urea, ammonia or nitrates as a nitrogen source can be used in this invention.

[0030] Typically, the fermentation is initiated with about 6 to 12 grams yeast extract per liter. More yeast extract can be added as required. A typical fermentation run requires from about 8 to 15 grams yeast extract per liter over the course of the run. Accordingly, that amount of yeast extract can be added initially with a reduced need for further additions. The precise amount can be determined by those of skill in the art. Generally, the ratio of glucose to yeast extract is from about 2:1 to about 25:1.

[0031] Cultivation can be carried out at any life-sustaining temperature. Generally, microorganisms such as *Cryptocodinium* or *Thraustochytrium* will grow at temperatures ranging from about 15°C to 34°C. Some fungi grow effectively at temperatures ranging from about 10°C to 80°C. Preferably, the temperature is maintained at about 20°C to 30°C. Strains which grow at higher temperatures are preferred, because they have a faster doubling time, thereby reducing total the fermentation time. Appropriate temperature ranges for other microorganisms are readily determined by those of skill in the art.

[0032] Cultivation can be carried out over a broad pH range, typically from about pH 5.0 to 9.0. Preferably, a pH range of from about 6.0 to about 7.0 is used for the growth phase. A base, such as KOH or NaOH, is used to adjust the media pH prior to inoculation. During the later stages of the fermentation, the pH of the culture medium can increase or decrease as nutrients are utilized. If desired, the pH can be adjusted during the fermentation to correct alkalinity or acidity during the growth phase by adding an appropriate acid or base.

[0033] Production of the microbial cell oil is induced in the microorganisms by the induction of a stationary phase by allowing the culture to reach a phase of nitrogen depletion or phosphate depletion or by allowing the pH of the culture to rise. Yeast extract deficiencies can be caused by providing only a limited amount of yeast extract such that the medium is depleted of its nitrogen source, while available glucose levels remain adequate for growth. It is the carbon source to nitrogen source ratio which promotes the efficient production of the single cell oil. Using glucose and yeast extract as examples, a preferred ratio of carbon source to nitrogen source at the time of inoculation is about 10 to 15 parts glucose to 1 part yeast extract. Similar ratios for other carbon and nitrogen sources can be calculated by those of skill in the art.

[0034] After induction of oil production, the culture is grown for about 24 additional hours. During this period the single cell oil containing DHA is being synthesized and oil droplets are visible inside the cells when they are observed using a microscope. Those of skill in the art can readily calculate the time of fermentation required to achieve the expected amount of cell biomass based upon the added amount of yeast extract. When that time has passed, the culture is grown for an additional 24 hours and harvested. In general, for example, the *Cryptothecodinium* or *Thraustochytrium* cells are cultivated for about 60 to about 90 hours, although this time is subject to variation.

[0035] Using the *Cryptothecodinium* strain designated as ATCC accession number 40750, as an example, from about 15 to 30% of the resulting biomass comprises extractable oil. Strain selection can increase this percentage. Preferably, the oil comprises greater than about 70% triglycerides having, in general, the following fatty acid composition.

15-20% myristic acid (C<sub>14:0</sub>)

15-25% palmitic acid (C<sub>16:0</sub>)

5-15% oleic acid (C<sub>18:1</sub>)

30-50% DHA (C<sub>22:6</sub>)

The crude oil is characterized by a yellow-orange color and is liquid at room temperature. Desirably, the oil contains at least about 20% DHA by weight, preferably about 40% DHA by weight, and most preferably at least about 50% DHA by weight.

[0036] The organisms are harvested by conventional means, known to those of skill in the art, such as centrifugation, flocculation or filtration, and can be processed immediately or dried for future processing. In either event, the oil can be extracted readily with an effective amount of solvent. Suitable solvents can be determined by those of skill in the art. However, preferred solvents include pure hexane and supercritical fluids, such as supercritical CO<sub>2</sub>. Certain lipophilic antioxidants such as  $\beta$ -carotene,  $\alpha$ -tocopherol, ascorbyl palmitate, and BHT can be added prior to extraction. These compounds help protect the oil from oxidation during the extraction and refining processes.

[0037] General extraction techniques using supercritical fluids have been developed for the extraction of oil from oil-rich plant seeds (McHugh et al. Supercritical Fluid Extraction, Butterworth, 1986). However, these standard methods generally are not applicable to the extraction of microorganisms. For example, spray dried algal cells have the consistency of flour, and the flow of supercritical CO<sub>2</sub> is restricted as the microorganism biomass is compressed. In addition, the cell walls of microalgae and fungi are chemically dissimilar to those of most seed oil material. In order to prevent the compression and allow efficient flow and extraction, the algal biomass can be mixed with from 0.1 to 5.0 parts of lipid free structural agent, such as Celite, or diatomaceous earth. In a 50 ml reaction vessel at 450 Atm. and 100°C., 86% of the oil was extracted from *C. cohnii* in 25 min-

utes, and 100% was extracted in 85 minutes.

[0038] If the extraction solvent is hexane, a suitable ratio of hexane to dry biomass is about 4 liters of hexane per kilogram of dry biomass. The hexane preferably is mixed with the biomass in a stirred reaction vessel at a temperature of about 20 to 50°C for about 2 hours. After mixing, the biomass is filtered and separated from the hexane containing the oil. Alternatively, a wet biomass paste that is from 30 to 35% solids can be extracted directly with more polar solvents, such as ethanol, isopropanol or mixtures of hexane and isopropanol.

[0039] The solvent is removed from the oil by distillation techniques known to those of skill in the art. Conventional seed oil processing equipment is suitable to perform the filtration, separation and distillation. Additional processing steps, known to those of skill in the art, can be performed if required or desirable for a particular application. These steps also will be similar to those involved in conventional vegetable oil processing and allow the separation of DHA-enriched polar lipid fractions.

#### ARA-Containing Oil Production

[0040] ARA producing fungi or algae are cultivated under suitable ARA-containing oil-producing cultivating conditions. If desired, the microorganism can be grown in a shake flask initially and then transferred to a fermentor. The composition of the growth medium can vary but always contains carbon and nitrogen sources. A preferred carbon source is glucose, amounts of which can range from about 10 to 200 grams glucose per liter of growth medium. Typically about 50 grams per liter are utilized for shaker flask culture. The amount can be varied depending upon the desired density of the final culture. Other carbon sources which can be used include molasses, high fructose corn syrup, hydrolyzed starch or any other low cost conventional carbon source used in fermentation processes. Additionally, lactose can be provided as a carbon source. Thus, whey permeate, which is high in lactose and is a very low cost carbon source, can be used as a substrate. Suitable amounts of these carbon sources can readily be determined by those of skill in the art. Usually, additional amounts of the carbon source needs to be added during the course of the fermentation.

[0041] Nitrogen typically is provided in the form of yeast extract at a concentration of from about 2 to about 15 grams per liter of growth medium. Preferably, about 8 to 10 grams per liter are provided. Other nitrogen sources can be used, including peptone, tryptone, corn steep liquor, etc. The amount to be added of these sources can easily be determined by those of skill in the art. Nitrogen can be added throughout the cultivation or in a batch mode, i.e. all at one time prior to cultivation.

[0042] After cultivation for 3 to 4 days at a suitable temperature, typically about 25 to 30°C, an amount of fungi or algae has grown which is sufficient for use as an inoculum in a conventional stirred tank fermentor or

an air lift fermentor. Fermentation can be carried out in batch, fed-batch, or continuous fermentation modes. The stirred tank fermentor is equipped with either a Rushton-type turbine impeller or, preferably, a marine-type axial impeller.

[0043] The fermentor is prepared by adding the desired carbon and nitrogen sources. For example, a 1.5 liter fermentor can be prepared by mixing about 50 grams of glucose and about 6 grams of yeast extract per liter of water. As previously discussed, other carbon or nitrogen sources or mixtures thereof can be used.

[0044] The reactor containing the nutrient solution should be sterilized by, for example, heating prior to inoculation as described above in the discussion of micro-organism cultivation for the production of DHA. After cooling to about 30°C, the inoculum can be added, and cultivation initiated. Gas exchange is provided by air sparging. The air sparging rate can vary, but preferably is adjusted to from about 0.5 to about 2.0 volumes of air per volume of fermentor per minute. Preferably the dissolved oxygen level is kept at from about 10% to about 50% of the air saturation value of the solution. Accordingly, adjustments in the sparging rate may be required during cultivation.

[0045] Agitation is desirable during fermentation. The agitation is provided by the impeller. Agitation tip speed preferably is set within the range of from about 50 cm per sec to about 500 cm per sec, preferably from about 100 to 200 cm per sec.

[0046] In general, the amount of inoculum used in a fermentation can vary. Typically, a logarithmically growing culture that is from about 2% to about 10% of the total volume of the medium in the fermentor can be used as an inoculum.

[0047] Nutrient levels should be monitored. When glucose levels drop below 5 grams per liter, additional glucose should be added. A typical cultivation cycle utilizes about 100 grams of glucose and about 15 grams of yeast extract per liter. It is desirable to deplete the nitrogen during the course of the cultivation as this enhances oil production by the fungi or algae. This is especially true when *M. alpina* is used as the production organism.

[0048] Occasionally, the culture will produce an excessive quantity of foam. Optionally, an antifoaming agent, such as those known to those of skill in the art, for example Mazu 310®, can be added to prevent foaming.

[0049] The temperature of cultivation can vary. However, those microorganisms which produce both ARA and EPA tend to produce less EPA and more ARA when cultivated at higher temperatures. For example, when *Mortierella alpina* is cultivated at less than 18°C, it begins to produce EPA. Thus, it is preferable to maintain the temperature at a level which induces the preferential production of ARA. Suitable temperatures are typically from about 25°C to about 30°C.

[0050] Preferably, cultivation continues until a desired biomass density is achieved. A desirable biomass is

about 15-40 grams per liter of the organism. Such a biomass typically is attained within 48 to 72 hours after inoculation. At this time, the organisms typically contain about 5 to 40% complex lipids, of which about 10 to 50% is ARA, and can be harvested.

[0051] Harvesting can be done by any suitable method such as filtration, centrifugation, or flocculation. Because of lower cost, filtration may be preferred.

[0052] After harvesting, the biomass can be extracted without drying. Optionally, the biomass can have any residual water removed, as by vacuum drying, fluid-bed drying or lyophilization, prior to extraction. If the water is removed, it is preferable to use nonpolar solvents to extract the ARA-containing oil. While any non-polar extract is suitable, hexane is preferred. Supercritical fluids such as CO<sub>2</sub> or NO, as discussed above also can be used for extraction of ARA-enriched oils from algae and fungi. Although fungi such as *M. alpina* are unexpectedly difficult to extract with CO<sub>2</sub>, as much as 89% of the oil of a fungal biomass can be recovered at temperatures above 90°C and pressures of 400 Atm. Alternatively, the wet biomass, which typically contains about 30 to 50% solids, can be crumbled and extracted directly using polar solvents such as ethanol, isopropyl alcohol or a mixture of hexane and isopropyl alcohol.

[0053] A preferable method of aqueous extraction involves mixing the biomass with the polar solvent isopropyl alcohol in a suitable reaction kettle. Such kettles are known. The use of three to six parts of solvent per part of biomass is desired. Most preferably, the mixing is done under nitrogen or with the addition of anti-oxidants such as β-carotene, α-tocopherol, ascorbyl palmitate or BHT to prevent the oxidation of the ARA in the lipid extract.

[0054] The solvent is removed from the oil as discussed in the section above regarding the production of a DHA-containing oil. Additional steps to further purify the oil also can be performed.

[0055] Yields can vary from about 5-50 grams of ARA-containing oil per 100 grams of dried biomass. In the case of *M. alpina*, 10 to 50 grams of triglyceride per 100 grams of dry biomass can be obtained. In the case of *Ochromonas*, 5 to 20 grams of triglyceride per 100 grams of biomass can be obtained.

[0056] Preferably the oil from *M. alpina* comprises greater than about 70% triglycerides having, in general, the following fatty acid composition:

- 5 - 15% palmitic acid
- 15 - 20% stearic acid
- 5 - 10% oleic acid
- 6 - 10% linoleic acid
- 2 - 10% linolenic acid
- 2 - 10% dihomo-gamma linolenic acid
- 40 - 50% arachidonic acid

### Administration of DHA and ARA Containing Oils

**[0057]** In accordance with this invention, DHA-containing microbial oils, ARA-containing microbial oils or suitable combinations of these oils are administered to patients affected by a neurological disorder characterized by depressed levels of DHA and/or ARA in the blood or tissues in comparison to the levels found in healthy individuals. The specific course of treatment administered will be determined based on normalization of serum and erythrocyte DHA and ARA levels. These serum levels of DHA and ARA are thought to reflect the long chain polyunsaturated fatty acid compositions of neurological membranes. In some cases, serum levels of ARA and DHA may need to be increased to 4 to 5 times the levels which are considered to be normal in the general population in order to see a therapeutic effect. Patients suffering from disorders involving such conditions as retinitis pigmentosa or senile dementia may respond to the administration of DHA-containing oil alone, while patients suffering from conditions such as adrenoleukodystrophy, diabetes-induced neuropathy or schizophrenia may respond more favorably to the administration of a combination of a DHA-containing oil and an ARA-containing oil. Still other patients may benefit from the administration of an ARA containing microbial oil alone.

**[0058]** The course of treatment can be followed by measuring levels of the fatty acid(s) of interest in the serum of treated patients. For some patients it will be possible to follow the normalization of DHA or ARA levels in neural tissue by measuring the levels of DHA and ARA in erythrocytes or in serum lipids during treatment.

**[0059]** Although the DHA- and/or ARA-containing oils can be administered to patients directly, more commonly they will be combined with one or more pharmaceutically acceptable carriers and, optionally, other therapeutic ingredients. Acceptable carriers are carriers which are compatible with the other components of the formulation and not deleterious to the patient.

**[0060]** Formulations include those suitable for oral, nasal, topical or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. It will be appreciated that the preferred formulation can vary with the condition and age of the patient. The formulations conveniently can be presented in unit dosage form, e.g., emulsions, tablets, and sustained release capsules, and can be prepared by any suitable pharmaceutical method.

**[0061]** Formulations of the present invention suitable for oral administration can be presented as discrete units such as capsules or tablets, each of which contains a predetermined amount of DHA or ARA oil or a predetermined amount of a suitable combination of DHA and ARA oils. These oral formulations also can comprise a solution or a suspension in an aqueous liquid or a non-aqueous liquid. The solution can be an emulsion such as an oil-in-water liquid emulsion or a water-in-oil liquid

emulsion. The oils can be administered by adding the purified and sterilized liquids to a prepared enteral formula which is then placed in the feeding tube of a patient who is unable to swallow.

**[0062]** In one preferred embodiment, the DHA or ARA microbial oil is incorporated into gel capsules such as those described in Example 6. However, it will be recognized that any known means of producing gel capsules can be used in accordance with the present invention.

**[0063]** Compressed tablets can be prepared by compressing or molding the microbial oil(s) in a suitable machine. The oil(s) can be mixed with dry inert accessory ingredients such as carboxymethyl cellulose. The tablets optionally can be coated or scored and can be formulated so as to provide slow or controlled release of the active ingredient therein.

**[0064]** Other formulations suitable for topical administration include lozenges comprising DHA oil, ARA oil or a combination thereof in a flavored basis, usually sucrose and acacia or tragacanth.

**[0065]** Formulations suitable for topical administration to the skin can be presented as ointments, creams and gels comprising the DHA and/or ARA oil(s) in a pharmaceutically acceptable carrier. A preferred topical delivery system is a transdermal patch containing the oil to be administered.

**[0066]** In formulations suitable for nasal administration, the carrier is a liquid, such as those used in a conventional nasal spray or nasal drops.

**[0067]** Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which optionally can contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which can include suspending agents and thickening agents. The formulations can be presented in unit-dose or multi-dose containers. A preferred embodiment of the present invention includes incorporation of the DHA and/or ARA oil(s) into a formulation for providing parenteral nutrition to a patient.

**[0068]** The microbial oil compositions of the present invention need not be administered as a pharmaceutical composition. They also can be formulated as a dietary supplement, such as a vitamin capsule or as food replacement in the normal diet. The microbial oils can be administered as a cooking oil replacement formulated so that in normal usage the patient would receive amounts of DHA and/or ARA sufficient to elevate the concentrations of these fatty acids in the serum and in membranes of affected neural tissues to normal or near-normal levels. A special emulsion type margarine could also be formulated to replace butter or ordinary margarine in the diet. The single cell microbial oils could be added to processed foods to provide an improved source of  $\omega$ -3 and  $\omega$ -6 unsaturated fatty acids. The oil can be microencapsulated using gelatin, casein, or oth-



er suitable proteins using methods known in the art, thereby providing a dry ingredient form of the oil for food processing. Such methods of administration can be preferred in the case of a person known to have a genetic predisposition to a disorder associated with a DHA or ARA metabolic deficiency such as a neurodegenerative disease, for example Huntington's disease or Alzheimer's disease. Providing such an individual with a dietary replacement can provide a significant prophylactic effect, delaying the onset of symptoms of a particular disorder. The administration of the long chain polyunsaturated fatty acids DHA and ARA offer a significant advantage over merely obtaining linoleic and linolenic acid, the precursors of these fatty acids, from standard foods or specialty oils such as primrose or borage oil. The administered DHA and ARA are already present in their active forms so that the patient is not required to metabolize dietary precursors. This results in the effective doses which are significantly lower than those of the precursors which would be required to produce the therapeutic effect.

**[0069]** It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention can include other suitable agents such as flavoring agents, preservatives and antioxidants. In particular, it is desirable to mix the microbial oils with an antioxidant to prevent oxidation of the DHA or ARA. Such antioxidants would be food acceptable and could include vitamin E, carotene, BHT or other antioxidants known to those of skill in the art.

**[0070]** The daily dose of the compositions of the present invention to be provided to a patient will depend upon the extent of the DHA and/or ARA deficit identified by serum lipid analysis prior to the introduction of the therapy. Typically, the initial dose provided to a patient of greater than 50 pounds will be in the range of about 50 mg DHA to 5000 mg DHA per day. A preferred maintenance dose is about 500 mg DHA per day. For example, if the DHA oil to be used is 50% enriched in DHA, such a dose would correspond to the addition of about 1000 mg of oil per day.

**[0071]** The daily dose of ARA provided to the patient of greater than 50 pounds will be 50 mg ARA to 5000 mg per day. A preferred maintenance dose would be 500-1000 mg per day. If the ARA oil to be used is 50% enriched in ARA, such a dose would correspond to the addition of about 1000-2000 mg of ARA oil per day. Doses of a suitable combination of the DHA and ARA containing oils will be 1000 mg of DHA and 1000 mg of ARA per day.

**[0072]** Desirably, the patient's serum fatty acid profiles are reviewed after about four weeks of this daily therapy. Subsequent doses then can be modified in response to the observed level of plasma lipid or red blood cell DHA and ARA and in response to observed clinical responses to the therapy. Patients with peroxisomal disorders can have red blood cell levels of DHA of only 1-3  $\mu\text{g}$  DHA per ml. of plasma. Normal target values range

from about 10 to 30  $\mu\text{g}$  of DHA per ml of plasma. Normal target values of circulating ARA range from about 75 to about 120  $\mu\text{g}$  ARA per ml. of plasma. Once normalized level(s) of the circulating fatty acid(s) of interest have been achieved, the daily dose of oil(s) can be modified to maintain the circulating DHA and/or ARA at a desirable level.

**[0073]** As noted above, in order to treat certain neurological disorders, it may be desirable to raise the level of circulating DHA and/or ARA in the blood to 4 to 5 times normal levels. The levels of circulating DHA and ARA, therefore, can be raised to about 120-150  $\mu\text{g}/\text{ml}$  and about 480-600  $\mu\text{g}/\text{ml}$ , respectively.

**[0074]** Although not wanting to be bound by any specific theory, it is the inventor's belief that the administration of DHA is effective for treating neurological disorders because of its ability to regulate calcium uptake by neuronal cells. A depolarization of the neuronal cell results in elevated levels of intracellular calcium, causing the activation of a phospholipase and resulting in the release of free DHA from the cell membrane. This free DHA acts as a calcium channel blocker, thereby limiting calcium entry into the cell. Thus, the level of DHA present in the neuronal cell membrane, and thereby available for activation-induced release of these long chain polyunsaturated fatty acids, may control intracellular calcium levels. If a deficiency of DHA exists, intracellular calcium levels rise, and the production of amyloid plaque protein is stimulated. Furthermore, high intracellular calcium stimulates the phosphorylation of the microtubule associated tau protein, resulting in the development of neurofibrillar tangles.

**[0075]** Serum lipids are the most probable source of the DHA and ARA incorporated into neuronal cells, since serum lipids act as the transport or carrier system for fatty acids in general. Studies in animals and in humans have shown that high levels of DHA and ARA in the serum are correlated with high levels of DHA and ARA in the brain. Therefore, elevating the DHA and ARA concentrations in the composition of total serum lipids, by providing supplemental dietary microbial oils enriched in these components, should increase the delivery of DHA and ARA to target neuronal tissues.

**[0076]** The role of ARA in neuronal function is less clear, although it too is a major component of neurological membranes. Many neurological disorders exhibit a deficiency of both DHA and ARA. The object of this invention is to supplement levels of both these components, using DHA and ARA from microbial oil to normalize both of these important fatty acids. The supplementation of DHA and ARA without any significant quantities of EPA is an important aspect of this invention, as the EPA levels in neurological tissues generally are low and supplementation with EPA will depress ARA levels, and may be contraindicated in certain instances. Therapeutic administration of the DHA oil in combination with the ARA oil may be beneficial in maintaining or establishing a ratio of  $\omega$ -3 to  $\omega$ -6 long chain polyunsaturated fatty acid

in the body comparable to that in normal healthy individuals.

[0077] The present invention having been generally described, reference is had to the following nonlimiting specific examples.

#### Example 1

[0078] A medium of one half strength artificial seawater made by combining 4.3 kg of Instant Ocean® with 230 liters of tap water was loaded into a 350 liter stirred tank fermentor. The fermentor containing the medium was sterilized and cooled to 28°C. 6.8 liters of concentrated yeast extract at a concentration of 400 grams per liter, 12.5 liters of glucose syrup at a concentration of 400 grams per liter and 30 liters of *C. cohnii* inoculum from a seed fermentor at a concentration of  $10^6$  cells per ml or a biomass density of about 1.3 grams per liter were added to the medium. Agitation was set at a tip speed of 73 cm per sec and aeration was set at 1 VVM, which is equivalent to 280 liters per minute. An additional 12 liters of glucose syrup was added after about 44 hours and another 43 liters was added over the next 32 hours. Thus, 67.5 liters of glucose syrup were added in total.

[0079] To maintain the dissolved oxygen at greater than 20%, the agitation tip speed was increased to 175 cm per second at 44 hours and to 225 cm per sec at 55 hours. At 76 hours, the tip speed was decreased to 150 cm per second. The culture was grown for an additional time sufficient to convert the final charge of glucose into cellular oil, then harvested. The harvested cells were dried to about a 4% moisture content. Hexane was added to the dried biomass and stirred in a glass kettle for 2 hours at 25°C. A rotary evaporator was used to remove the hexane, producing about 700 grams of crude DHA-containing oil.

#### Example 2

[0080] Sixty kg of yeast extract, 45 kg of NaCl, 12.3 kg of  $MgSO_4 \cdot 7H_2O$ , and 0.9 kg of  $CaCl_2 \cdot 2H_2O$  in 7,000 liters of water were loaded into a 15,000 liter fermentor. After this solution was sterilized, 3,000 liters of a sterilized glucose solution at a concentration of 650 kg of glucose per 3,000 liters of volume was added. The initial pH of the medium was 6.3, the temperature was 28°C, aeration was 0.5 to 1.0 VVM, the vessel back pressure was set to 0.2 bar, and the agitation tip speed was set to 120 cm per seconds before the vessel was inoculated with 300 liter of an inoculum culture of *C. cohnii* which had attained a cell density of about  $60 \times 10^6$  cells per ml, which is equivalent to 4 to 5 grams of dry weight of biomass per liter of culture in the inoculum tank. During the course of the fermentation, a food grade antifoam, such as Dow 1520 was added as needed and the pH was held at 6.3 using either 8 N  $H_2SO_4$  or 4 N NaOH as needed. The dissolved oxygen level was maintained at

greater than 20% of air saturation by increasing the vessel back pressure and agitation. Additional glucose additions were required at 93 hours and at 111 hours to maintain the glucose levels above 5 grams per liter. At 119 hours, the fermentor was cooled to 17°C and the fermentation broth was processed through a centrifuge. A 608 kg slurry containing 25% solids was recovered. The slurry was spray dried, producing about 150 kg dry algal powder which contained about 30 to 40 kg of oil with a DHA content of 40 to 45%.

[0081] The dry algal powder was extracted with hexane using standard vegetable oil extraction equipment and methods. Following the removal of the solvent, the crude oil was degummed by the adding water at 50°C. The degummed oil was collected by centrifugation and refined by mixing with caustic base and phosphoric acid at 55°C for one hour. The refined and degummed oil was then collected by centrifugation and gently bleached at 90°C by the addition of citric acid and bleaching clay. Filtration of the bleaching clay produced the refined, bleached oil (RB-oil) with a peroxide value of less than 5 mEq per kg. The RB-oil was deodorized by high vacuum short path distillation and the resulting deodorized RB-oil (RBD-oil) was then ready for encapsulation, tableting, or bulk shipping. The resulting oil had a peroxide value less than 1 mEq per kg, a free fatty acid content of less than 0.05%, a DHA content of 45 to 47%, and an iodine number of about 200.

#### Example 3

Preparation of *Thraustochytrium aurum* lipid

[0082] 2.5 grams of NaCl, 5 grams of  $MgSO_4 \cdot 7H_2O$ , 1 gram of KCl, 0.1 grams of  $KH_2PO_4$ , 0.2 grams of  $CaCO_3$ , 0.2 grams of  $(NH_4)_2SO_4$ , 2.0 grams of sodium glutamate in 1 liter of water were loaded into a 1.7 liter stirred tank fermentor. After the tank was sterilized, a sterile solution containing 10 µgrams of thiamine-HCl, 0.1 grams of  $NaHCO_3$ , and 10 µgrams of vitamin  $B_{12}$  was added- thiamine  $B_{12}$  followed by the addition of 150 ml of sterile 30% glucose and 50 ml of sterile 10% yeast extract. The pH was adjusted to 6.0, the sparging was adjusted to 1.0 VVM, and agitation was adjusted to 300 rpm before inoculation with 100 ml of a 5-day old shake flask culture of *Thraustochytrium aurum* grown in the same medium. The culture was harvested after 9 days to yield about 4 grams dry weight of biomass. The DHA content of the lipid in the biomass is 10 to 15%.

#### Example 4

Preparation of *Pythium insidiosum* lipid

[0083] In an 80 liter (gross volume) fermentor, 51 liters of tap water, 1.2 kg glucose, 240 grams of yeast extract and 15 ml of MAZU 210S® antifoam were combined. The fermentor was sterilized at 121°C for 45 minutes.

An additional 5 liters of condensate water were added during the sterilization process. The pH was adjusted to 6.2, and approximately 1 liter of inoculum at a cell density of 5 to 10 grams per liter of *Pythium insidiosum* (ATCC #28251) then was added. The agitation rate was adjusted to 125 RPM corresponding to a tip speed of 250 cm per second and the aeration rate was set at 1 SCFM (standard cubic feet per minute). At hour 24 in the operation the aeration rate was increased to 3 SCFM. At hour 28 an additional 2 liters of a 50% by weight glucose syrup were added. At hour 50 the fermentor was harvested, resulting in a yield of about 2.2 kg wet weight of biomass, which was approximately 15 grams of dry weight per liter of culture. The harvested biomass was squeezed to a high solids cake, comprising approximately 50% solids, on a suction filter before it was freeze dried. The dried biomass was ground with a mortar and pestle and extracted with 1 liter of hexane per 200 grams of dry biomass at room temperature under continuous stirring for 2 hours. The mixture then was filtered and the filtrate evaporated, yielding about 5 to 6 grams of crude oil per 100 grams of dry biomass. The biomass was reextracted with 1 liter of ethanol per 20 grams of dry biomass for 1 hour at room temperature, filtered, and the solvent evaporated, yielding an additional 22 grams of crude oil per 100 grams of dry biomass. The second fraction was predominantly phospholipids whereas the first fraction contained a mixture of phospholipids and triglycerides. The combined fractions produced an oil containing about 30 to 35% arachidonic acid and no detectable EPA.

#### Example 5

##### Preparation of *Mortierella alpina* lipid

[0084] A 7,500 liter fermentor was filled with 4,500 liters of water and charged with 225 kg dextrose, 27 kg yeast extract and 450 grams of antifoam (Dow 1520). The pH was adjusted to 5.0 and the medium was sterilized for 60 minutes at 121°C. After sterilization and cooling to 28°C, the pH was adjusted to 5.5 with NaOH, the aeration adjusted to 0.25 VVM, the back pressure set at 0.2 bar, and the agitation of the A315 impellers was set at a speed of 80 cm per second. The culture was inoculated with 180 liters of a 20 hour old seed culture of *Mortierella alpina* at 2.2 grams per liter. The pH was allowed to fluctuate until 37 hours into the run at which time it was controlled at 6.5. The agitation was increased to 110 cm per second at 26 hours during the peak oxygen demand, but it was returned to 80 cm per second at 32 hours. At 123 hours the tank was harvested using a Bock basket centrifuge fitted with a 40 micron bag. The material was then dried using a fluid bed drier and extracted with hexane as in Example 2. The fermentation yielded 17 kilograms of a crude oil with an ARA content of 45%.

#### Example 6

[0085] DHA-enriched oil prepared in accordance with Example 1 or 2 was prepared for oral use by either encapsulating or tableting. Clear sealed gelatin capsules of 1 gram per capsule were prepared by conventional manufacturing methods. Banded gel caps containing one oil or a mixture of oils were prepared by allotting 250 µl of oil in the gel cap bottoms using a positive displacement manifold pipetter. With this method weight accuracy of ±3-5% was attained. The tops then were placed over the gels caps and were banded with dyed gelatin using a capsule banding machine. Alternatively, the gel cap bottoms were filled with carboxymethylcellulose and 120 µl of oil was pipetted directly onto this binder where it was adsorbed, preventing any leakage. The tops were placed over the gel caps and were banded with dyed gelatin using a capsule banding machine. Alternatively, the carboxymethylcellulose could be mixed with oil at a ratio of three parts carboxymethylcellulose to one part oil in a separate container and then pressed into tablets using a tablet press.

[0086] The procedure was repeated using ARA-enriched oil produced in accordance with Examples 4 and 5.

#### Example 7

[0087] Crude microbial oils produced from microorganisms such as those described in examples 1, 2, 3, 4 and 5 were processed using conventional vegetable oil processing methods. The oil was degummed to remove phosphatides by mixing with water at 50°C, then removing the water and gum mixture by centrifugation. The oil was refined to remove the free fatty acids by mixing with caustic base followed by phosphoric acid at 55°C, then removing the water, fatty acid mixture by centrifugation. The processed microbial oil was bleached by mixing with citric acid and standard bleaching clay at 90°C before filtration to remove the spent clay and any remaining polar particles in the oil. In some cases the oil was deodorized using either a high vacuum distillation or a counter current steam stripping deodorizer resulting in the production of the final, refined, bleached and deodorized oil (RBD oil). Specifications of the oils flowing through this process typically gave a peroxide value of less than 2 mEq per kilogram and a free fatty acid level of less than 0.05%. These specifications are typical for standard vegetable oils and the microbial oils are used in this state in place of vegetable oil in the preparation of margarine, shortenings, spoonable dressings, liquid dressings, or as the oil component of other manufactured food products. The microbial oils are highly enriched in long chain polyunsaturated fatty acids, in particular DHA and ARA, and are diluted by at least one part per ten parts of a conventional oil chosen for the particular product being prepared. For incorporation into chocolate products, the oil is diluted with co-

coa butter. For use as a shortening or baking product, the oil is diluted with coconut or palm oils. For use as a salad dressing, the oil is diluted with standard salad oils such as canola, soy, safflower or corn oil.

#### Example 8

[0088] A patient exhibiting the symptoms of Alzheimer's disease is treated with DHA-enriched oil from *C. cohnii* by administering 1 to 3 capsules containing 1 gram of DHA single cell microbial oil, containing 50% DHA, per day. The patient's serum levels of DHA and plasmalogens are routinely monitored during the period of the administration in order to determine when the serum levels of these two substances are normalized. Serum DHA levels of two-times the American normal are preferred.

#### Example 9

[0089] A patient with a peroxisomal disorder was administered 0.5 ml (500mg) of DHA oil at a concentration of 200-250 mg of DHA, directly into the gastrostomy feedings consisting of Sustical (Ross Laboratories) once a day. The patient's serum DHA and plasmalogen levels were routinely monitored during the period of administration. Within six weeks of the initiation of the treatment, the patient's DHA levels improved from 1.85  $\mu\text{g/ml}$  of plasma to 13.6  $\mu\text{g/ml}$  of plasma. Normal levels are  $19.0 \pm 6.4 \mu\text{g/ml}$ . Unexpectedly the patient's plasmalogen levels are normalized as well. With the treatment of the DHA oil alone, however the serum ARA-levels remained relatively unchanged at about 50% of normal levels.

#### Example 10

[0090] A patient suffering from a form of senile dementia whose serum DHA and ARA levels are depressed is administered 1 to 3 one gram capsules per day, each of which contains DHA and ARA oils at a ratio of about 2:1 ARA to DHA and an overall dose of DHA of 500 mg per day and ARA of 1000 mg per day. The patient's serum lipids are rechecked in four weeks. If the serum DHA and ARA levels are less than five times the normal levels (i.e., if serum DHA is less than 100  $\mu\text{g}$  per ml of plasma and serum ARA less than 500  $\mu\text{g}$  per ml plasma), and the symptoms persist, the patient should remain on the same dose regimen until the serum DHA and ARA reach the desired levels and/or symptoms are alleviated. The dose then can be lowered until the symptoms once again appear or until the serum ARA and DHA levels are in the normal range.

#### Example 11

[0091] A patient with schizophrenia is administered 1 to 9 one gram capsules per day, each of which contains

a balance of DHA and ARA oils providing an ARA/DHA ratio of about 2:1 and an overall dose of DHA of 1000 mg/day and ARA of 2000 mg/d. The patient's serum lipids are rechecked in four weeks. If the serum DHA and ARA levels are less than five times normal levels (i.e., DHA less than 100  $\mu\text{g/ml}$  plasma and ARA less than 500  $\mu\text{g/ml}$  plasma) and the symptoms persist the patient should remain on the same dose regimen until the desired levels are reached and/or symptoms are alleviated. Once the symptoms of the neuropathy are relieved at the given dose, the maintenance dose can be titrated down until the symptoms once again appear or until the serum ARA and DHA levels are in the normal range.

#### Example 12

[0092] Blood lead levels of greater than 10  $\mu\text{g}$  per deciliter are considered "neurologically significant" by the U.S. Centers for Disease Control and Prevention. Patients tested and found to have blood lead levels in excess of 10  $\mu\text{g}$  per deciliter are administered 1 - 3 one gram gel capsules per day, each of which contains a balance of DHA and ARA oils providing an ARA/DHA ratio of about 2:1 and an overall dose of DHA of 250 mg/day and ARA of 500 mg/d. The patient's serum lead levels, plasma fatty acids and plasmalogen levels are rechecked in four weeks. If the serum DHA and ARA levels are less than five times the normal levels (i.e., DHA less than 100  $\mu\text{g/ml}$  plasma and ARA less than 500  $\mu\text{L/ml}$  plasma) and the symptoms or elevated lead levels persist, the patient should remain on the same dose regimen until the desired fatty acid levels are achieved and/or the symptoms are alleviated. Once the serum ARA and DHA levels are greater than five times the normal level then the dose levels should be reduced. If the symptoms of the neuropathy are relieved or the serum lead levels are reduced at the given dose, then the maintenance dose may be titrated down until the symptoms once again appear or until the serum ARA and DHA levels are in the normal range.

#### Claims

1. Use of a composition comprising docosahexaenoic acid (DHA), in the manufacture of a medicament for the treatment, alleviation or prevention of the symptoms of an ailment selected from senile dementia and Alzheimer's disease, **characterised in that** the composition is substantially free of eicosapentaenoic acid (EPA).
2. The use according to claim 1, wherein the composition further comprises arachidonic acid (ARA).
3. The use of claim 2, wherein the DHA is supplied as a DHA-containing microbial oil DHA and/or the ARA is supplied as an ARA-containing microbial oil ARA.

4. The use of claim 3, wherein said DHA-containing microbial oil is produced by a microorganism which is a species of *Cryptocodinium*, *Thraustochytrium* or *Schizochytrium*.  
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5. The use of claim 3, wherein said ARA-containing microbial oil is produced by a microorganism which is a species of *Porphyridium*, *Ochromonas*, *Mucor*, *Pythium* or *Mortierella*.  
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6. The use of claim 2, wherein the DHA-containing microbial oil comprises at least 20% DHA by weight and the ARA-containing microbial oil comprises at least 10% ARA by weight.  
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7. The use of claim 1 or claim 2, wherein the DHA-containing microbial oil DHA comprises at least 40% DHA by weight.
8. The use of claim 3, wherein said DHA-containing microbial oil and ARA-containing microbial oil are administered at a dosage concentration of about 50 to about 5000 mgs of DHA per day and about 50 to about 5000 mgs of ARA per day.  
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9. The use of claim 3, wherein said microbial oil DHA is administered at a dosage concentration of about 50 to about 5000 mgs of DHA per day.
10. The use of any preceding claim, wherein said composition is administered orally. 30
11. The use of any preceding claim, wherein said composition is administered in a capsule, a tablet or an emulsion. 35
12. The use of any preceding claim, wherein said composition is administered as a component of a dietary supplement, preferably a vitamin capsule or a food replacement. 40
13. The use according to claim 2, wherein the composition further comprises a concentration effective to maintain the circulating DHA and/or ARA levels at about 10 to 30 micrograms of DHA per ml of plasma and about 75 to about 120 micrograms of ARA per ml of plasma. 45

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European Patent  
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# EUROPEAN SEARCH REPORT

Application Number  
EP 04 07 5413

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
E	EP 0 622 463 A (KAWASAKI STEEL CO) 2 November 1994 (1994-11-02) * abstract * * page 2, line 1 - line 29; claims; examples *	1,7, 10-12	A61K35/70 A61K35/80 A61K31/202 A61K31/232 A61P25/28
X	EP 0 342 795 A (TAIYO GYOGYO KABUSHIKI KAISHA) 23 November 1989 (1989-11-23) * the whole document *	1-13	
X	EP 0 234 733 A (EFAMOL LTD) 2 September 1987 (1987-09-02) * abstract * * page 2, line 43 - page 3, line 13; claims 6-8; examples *	1-13	
X	EP 0 454 102 A (EFAMOL HOLDINGS) 30 October 1991 (1991-10-30) * whole document, in particular page 7, lines 42-43; claim 8 *	1-13	
X	EP 0 347 056 A (EFAMOL HOLDINGS) 20 December 1989 (1989-12-20) * whole document, in particular page 5, lines 25-29; page 7, lines 27-28 *	1-13	TECHNICAL FIELDS SEARCHED (Int.Cl.7) A61K A61P
X	PATENT ABSTRACTS OF JAPAN vol. 014, no. 047 (C-0682), 29 January 1990 (1990-01-29) & JP 01 279827 A (TAIYO FISHERY CO LTD), 10 November 1989 (1989-11-10)	1,7, 10-12	
Y	* abstract *	2-6,8,9, 13	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 26 March 2004	Examiner Hoff, P
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			



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## EUROPEAN SEARCH REPORT

Application Number  
EP 04 07 5413

DOCUMENTS CONSIDERED TO BE RELEVANT				
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)	
Y	DATABASE WPI Section Ch, Week 9019 Derwent Publications Ltd., London, GB; Class B05, AN 90-142346 XP002068901 & JP 02 086789 A (NIPPON OILS & FATS CO LTD) * abstract *	2-6,8,9,13		
D,A	WO 92 12711 A (MARTEK CORP) 6 August 1992 (1992-08-06) * abstract; claims; examples 1-3 *	1-13		
A	WO 91 11918 A (MARTEK CORP) 22 August 1991 (1991-08-22) * abstract * * page 13, line 7 - page 14, line 18; claims; examples *	1-13		
A	WO 92 13086 A (MARTEK CORP) 6 August 1992 (1992-08-06) * abstract * * page 15, line 30 - page 16, line 20; claims; examples *	1-13		TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	EP 0 218 460 A (EFAMOL LTD) 15 April 1987 (1987-04-15) * the whole document *	1-13		
A	MARTINEZ M: "DEVELOPMENT PROFILES OF POLYUNSATURATED FATTY ACIDS IN THE BRAIN OF NORMAL INFANTS AND PATIENTS WITH PEROXISOMAL DISEASES: SEVERE DEFICIENCY OF DOCOSAHEXAENOIC ACID IN ZELLWEGER'S AND PSEUDO-ZELLWEGER'S SYNDROMES" WORLD REVIEW OF NUTRITION AND DIETETICS, vol. 66, 1991, pages 87-102, XP000603946 * the whole document *	1-13		
The present search report has been drawn up for all claims				
Place of search THE HAGUE		Date of completion of the search 26 March 2004	Examiner Hoff, P	
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document				



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Office

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Application Number  
EP 04 07 5413

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	M. MARTINEZ: "TREATMENT WITH DOCOSAHEXAENOIC ACID FAVORABLY MODIFIES THE FATTY ACID COMPOSITION OF ERYTHROCYTES IN PEROXISOMAL PATIENTS" NEW DEVELOPMENTS IN FATTY ACID OXIDATION. ED. P: COATES, vol. 375, 1992, pages 389-397, XP002068900 * the whole document *	1-13	
A	D. BATES ET AL.: "A DOUBLE-BLIND CONTROLLED TRIAL OF LONG CHAIN N-3 POLYUNSATURATED FATTY ACIDS IN THE TREATMENT OF MULTIPLE SCLEROSIS" JOURNAL OF NEUROLOGY, NEUROSURGERY AND PSYCHIATRY, vol. 52, 1989, pages 18-22, XP002016094 * the whole document *	1-13	
P,A	M. MARTINEZ ET AL.: "DOCOSAHEXAENOIC ACID-A NEW THERAPEUTIC APPROACH TO PEROXISOMAL-DISORDER PATIENTS" NEUROLOGY, vol. 43, no. 7, July 1993 (1993-07), pages 1389-1397, XP002016092 * the whole document *	1-13	
P,A	EP 0 599 576 A (SCOTIA HOLDINGS PLC) 1 June 1994 (1994-06-01) * the whole document *	1-13	
The present search report has been drawn up for all claims			
Place of search <b>THE HAGUE</b>		Date of completion of the search <b>26 March 2004</b>	Examiner <b>Hoff, P</b>
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			



**ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.**

EP 04 07 5413

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

26-03-2004

Patent document cited in search report		Publication date		Patent family member(s)		Publication date	
EP 0622463	A	02-11-1994	CA	2121986 A1		27-10-1994	
			CN	1101034 A		05-04-1995	
			EP	0622463 A2		02-11-1994	
			JP	7008268 A		13-01-1995	
-----							
EP 0342795	A	23-11-1989	EP	0342795 A2		23-11-1989	
			JP	2049723 A		20-02-1990	
			JP	2524217 B2		14-08-1996	
-----							
EP 0234733	A	02-09-1987	AT	69378 T		15-11-1991	
			AU	593317 B2		08-02-1990	
			AU	6767587 A		30-07-1987	
			DE	3774449 D1		19-12-1991	
			EP	0234733 A1		02-09-1987	
			ES	2040242 T3		16-10-1993	
			GR	3003120 T3		17-02-1993	
			HK	127893 A		26-11-1993	
			IE	59606 B1		09-03-1994	
			JP	62185018 A		13-08-1987	
			SG	114293 G		21-01-1994	
			US	4753964 A		28-06-1988	
			US	4810497 A		07-03-1989	
			ZA	8700560 A		30-09-1987	
-----							
EP 0454102	A	30-10-1991	AT	87825 T		15-04-1993	
			AU	618814 B2		09-01-1992	
			AU	3597489 A		14-12-1989	
			AU	633442 B2		28-01-1993	
			AU	7943491 A		12-09-1991	
			CA	1334004 C		17-01-1995	
			DE	68905863 D1		13-05-1993	
			DE	68905863 T2		26-08-1993	
			EP	0347056 A1		20-12-1989	
			EP	0454102 A2		30-10-1991	
			ES	2053990 T3		01-08-1994	
			IE	63303 B1		05-04-1995	
			JP	2032017 A		01-02-1990	
			JP	2796838 B2		10-09-1998	
			KR	129666 B1		09-04-1998	
			NZ	229423 A		28-10-1992	
			NZ	239126 A		27-07-1997	
			US	4977187 A		11-12-1990	
			US	5120760 A		09-06-1992	
			ZA	8904380 A		28-02-1990	
-----							
EP 0347056	A	20-12-1989	AT	87825 T		15-04-1993	

**ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.**

EP 04 07 5413

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

26-03-2004

Patent document cited in search report		Publication date	Patent family member(s)	Publication date	
EP 0347056 A		AU	618814 B2	09-01-1992	
		AU	3597489 A	14-12-1989	
		AU	633442 B2	28-01-1993	
		AU	7943491 A	12-09-1991	
		CA	1334004 C	17-01-1995	
		DE	68905863 D1	13-05-1993	
		DE	68905863 T2	26-08-1993	
		EP	0347056 A1	20-12-1989	
		EP	0454102 A2	30-10-1991	
		ES	2053990 T3	01-08-1994	
		IE	63303 B1	05-04-1995	
		JP	2032017 A	01-02-1990	
		JP	2796838 B2	10-09-1998	
		KR	129666 B1	09-04-1998	
		NZ	229423 A	28-10-1992	
		NZ	239126 A	27-07-1997	
		US	4977187 A	11-12-1990	
		US	5120760 A	09-06-1992	
		ZA	8904380 A	28-02-1990	
-----					
JP 01279827	A	10-11-1989	NONE		
-----					
JP 2086789	A	27-03-1990	JP 2730081 B2	25-03-1998	
-----					
WO 9212711	A	06-08-1992	AT	200619 T	15-05-2001
			AU	661297 B2	20-07-1995
			AU	1239292 A	27-08-1992
			BR	9205526 A	19-04-1994
			CA	2101274 A1	25-07-1992
			DE	69231793 D1	23-05-2001
			DE	69231793 T2	08-11-2001
			DE	568606 T1	25-02-1999
			DK	568606 T3	23-07-2001
			EP	1092352 A2	18-04-2001
			EP	0568606 A1	10-11-1993
			ES	2157898 T3	01-09-2001
			GR	3036139 T3	28-09-2001
			IL	100733 A	31-12-1995
			IL	114253 A	13-07-1997
			JP	2731035 B2	25-03-1998
			JP	6505153 T	16-06-1994
			MX	9200320 A1	01-10-1992
			NZ	241359 A	26-08-1994
			OA	10348 A	19-10-2001
			RU	2093996 C1	27-10-1997
			SG	49307 A1	18-05-1998

**ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.**

EP 04 07 5413

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

26-03-2004

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9212711	A		WO 9212711 A1	06-08-1992
			US 5550156 A	27-08-1996
			US 5374657 A	20-12-1994
			ZA 9200452 A	28-10-1992
-----				
WO 9111918	A	22-08-1991	US 5407957 A	18-04-1995
			AT 165212 T	15-05-1998
			AU 660162 B2	15-06-1995
			AU 7330291 A	03-09-1991
			BR 9106038 A	02-02-1993
			CA 2076018 A1	14-08-1991
			DE 69129296 D1	28-05-1998
			DE 69129296 T2	05-11-1998
			DK 515460 T3	22-02-1999
			EP 0515460 A1	02-12-1992
			ES 2116288 T3	16-07-1998
			IL 97126 A	15-03-1995
			IL 111174 A	10-03-1998
			JP 2830951 B2	02-12-1998
			JP 5503425 T	10-06-1993
			JP 2003304894 A	28-10-2003
			WO 9111918 A1	22-08-1991
			US 5492938 A	20-02-1996
			US 5711983 A	27-01-1998
			US 5397591 A	14-03-1995
-----				
WO 9213086	A	06-08-1992	AT 196167 T	15-09-2000
			AU 661674 B2	03-08-1995
			AU 1235592 A	27-08-1992
			BR 9205519 A	01-03-1994
			CA 2101273 A1	25-07-1992
			DE 69231418 D1	12-10-2000
			DE 69231418 T2	13-06-2001
			DE 568608 T1	22-04-1999
			DK 568608 T3	09-10-2000
			EP 0568608 A1	10-11-1993
			EP 1001034 A1	17-05-2000
			ES 2149169 T3	01-11-2000
			GR 3034953 T3	28-02-2001
			IL 100732 A	29-06-1995
			JP 3229268 B2	19-11-2001
			JP 11151075 A	08-06-1999
			JP 6505384 T	23-06-1994
			KR 254300 B1	01-05-2000
			MX 9200301 A1	01-08-1992
			NZ 241358 A	27-09-1994

**ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.**

EP 04 07 5413

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

26-03-2004

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9213086	A		OA 9909 A	15-09-1994
			RU 2120998 C1	27-10-1998
			WO 9213086 A1	06-08-1992
			US 5658767 A	19-08-1997
			ZA 9200454 A	28-10-1992
-----				
EP 0218460	A	15-04-1987	AT 84414 T	15-01-1993
			AU 586286 B2	06-07-1989
			AU 6340786 A	09-04-1987
			CA 1279824 C	05-02-1991
			DE 3687501 D1	25-02-1993
			DE 3687501 T2	19-05-1993
			EP 0218460 A2	15-04-1987
			ES 2043601 T3	01-01-1994
			GR 3007043 T3	30-07-1993
			HK 129393 A	03-12-1993
			IE 59445 B1	23-02-1994
			JP 62096421 A	02-05-1987
			SG 115193 G	21-01-1994
			US 4826877 A	02-05-1989
			ZA 8607386 A	28-10-1987
-----				
EP 0599576	A	01-06-1994	AT 144706 T	15-11-1996
			AU 666782 B2	22-02-1996
			AU 5183093 A	09-06-1994
			AU 5232996 A	18-07-1996
			CA 2109777 A1	27-05-1994
			CN 1104494 A	05-07-1995
			DE 69305723 D1	05-12-1996
			DE 69305723 T2	03-04-1997
			DK 599576 T3	25-11-1996
			EP 0599576 A1	01-06-1994
			EP 0733360 A2	25-09-1996
			ES 2093935 T3	01-01-1997
			GR 3021692 T3	28-02-1997
			HK 114297 A	29-08-1997
			JP 6199663 A	19-07-1994
			NO 934266 A	27-05-1994
			NZ 250265 A	24-06-1997
			RU 2123844 C1	27-12-1998
			SG 47838 A1	17-04-1998
			US 5516800 A	14-05-1996
			ZA 9308835 A	02-08-1994
-----				